(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2002 (12.09.2002)

PCT

(10) International Publication Number
WO 02/070551 A2

(51) International Patent Classification[7]: C07K 14/47, A61K 38/17, A61P 25/02

(21) International Application Number: PCT/CA02/00257

(22) International Filing Date: 28 February 2002 (28.02.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/272,063 1 March 2001 (01.03.2001) US

(71) Applicant *(for all designated States except US)*: **MCGILL UNIVERSITY** [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ROSENBERG, Lawrence** [CA/CA]; 6507, Fern Road, Côte St-Luc, Québec H4V 1E4 (CA). **MAYSINGER, Dusica** [CA/CA]; 406, Pine Avenue, Montréal, Québec H2W 1S2 (CA).

(74) Agents: **OGILVY RENAULT** et al.; Suite 1600, 1981 McGill College Avenue, Montreal, Québec H3A 2Y3 (CA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/070551 A2

(54) **Title:** NEURITOGENIC COMPOUND AND USES THEREOF

(57) **Abstract:** The present invention relates to a neuritogenic compound for neurite outgrowth, which comprises the amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3), functional derivatives and/or fragments thereof and functional peptidometics thereof. There is also provided a method for repair and/or regeneration of peripheral nervous system in a patient.

NEURITOGENIC COMPOUND AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a neuritogenic compound derived from a peptide fragment of Islet Neogenesis Associated Protein (INGAP) for neurite outgrowth, including for repair and/or regeneration in the peripheral nervous system.

(b) Description of Prior Art

Using a model of partial pancreatic duct obstruction, the applicants have previously demonstrated that islet cell differentiation from progenitor cells associated with the ductal epithelium could be induced in the adult pancreas (Rosenberg, L. et al. (1983) *J. Surg. Res.* **35**, 63-72; Rosenberg, L. et al. (1990) *Surgery* **108**, 191-197; Rosenberg, L. (1998) *Microsc. Res. Tech.* **43**, 337-346) and that the newly formed islets functioned to reverse a diabetic state (Rosenberg, L. et al. (1988) *Diabetes* **37**, 334-341; Watkins, P. J. (1993) *Diabetes Med.* **10***(Suppl. 2)*, 77S-78S). Evidence obtained from parabiotic studies suggested that the induction of cell proliferation and differentiation was mediated by paracrine and/or autocrine mechanisms. Using classical protein chemistry techniques, a crude soluble tissue extract was isolated from the pancreas, and was shown to stabilize or reverse streptozotocin-induced diabetes. Subsequent investigations led to the isolation of a novel gene and protein product responsible for the bioactivity of the extract. This 175 amino acid protein was termed islet-neogenesis-associated protein, INGAP.

There was reported the identification of a gene, INGAP, that shows striking homology to the pancreatitis associated protein (PAP) family of genes (7-11). The predicted protein shares the carbohydrate recognition domain (CRD) of the calcium dependent C-type lectins. INGAP plays a role in stimulation of islet neogenesis, in particular, in beta cell regeneration from ductal cells.

The cDNA sequence of a mammalian INGAP is as follows:

```
CTGCAAGACA GGTACCATG ATG CTT CCC ATG ACC CTC TGT AGG ATG TCT TGG    52
                     Met Leu Pro Met Thr Leu Cys Arg Met Ser Trp
                      1               5                   10

ATG CTG CTT TCC TGC CTG ATG TTC CTT TCT TGG GTG GAA GGT GAA GAA   100
Met Leu Leu Ser Cys Leu Met Phe Leu Ser Trp Val Glu Gly Glu Glu
            15                  20                  25
```

```
TCT CAA AAG AAA CTG CCT TCT TCA CGT ATA ACC TGT CCT CAA GGC TCT    148
Ser Gln Lys Lys Leu Pro Ser Ser Arg Ile Thr Cys Pro Gln Gly Ser
         30                  35                  40

GTA GCC TAT GGG TCC TAT TGC TAT TCA CTG ATT TTG ATA CCA CAG ACC    196
Val Ala Tyr Gly Ser Tyr Cys Tyr Ser Leu Ile Leu Ile Pro Gln Thr
     45                  50                  55

TGG TCT AAT GCA GAA CTA TCC TGC CAG ATG CAT TTC TCA GGA CAC CTG    244
Trp Ser Asn Ala Glu Leu Ser Cys Gln Met His Phe Ser Gly His Leu
 60                  65                  70                  75

GCA TTT CTT CTC AGT ACT GGT GAA ATT ACC TTC GTG TCC TCC CTT GTG    292
Ala Phe Leu Leu Ser Thr Gly Glu Ile Thr Phe Val Ser Ser Leu Val
                 80                  85                  90

AAG AAC AGT TTG ACG GCC TAC CAG TAC ATC TGG ATT GGA CTC CAT GAT    340
Lys Asn Ser Leu Thr Ala Tyr Gln Tyr Ile Trp Ile Gly Leu His Asp
             95                 100                 105

CCC TCA CAT GGT ACA CTA CCC AAC GGA AGT GGA TGG AAG TGG AGC AGT    388
Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly Trp Lys Trp Ser Ser
        110                 115                 120

TCC AAT GTG CTG ACC TTC TAT AAC TGG GAG AGG AAC CCC TCT ATT GCT    436
Ser Asn Val Leu Thr Phe Tyr Asn Trp Glu Arg Asn Pro Ser Ile Ala
    125                 130                 135

GCT GAC CGT GGT TAT TGT GCA GTT TTG TCT CAG AAA TCA GGT TTT CAG    484
Ala Asp Arg Gly Tyr Cys Ala Val Leu Ser Gln Lys Ser Gly Phe Gln
140                 145                 150                 155

AAG TGG AGA GAT TTT AAT TGT GAA AAT GAG CTT CCC TAT ATC TGC AAA    532
Lys Trp Arg Asp Phe Asn Cys Glu Asn Glu Leu Pro Tyr Ile Cys Lys
                160                 165                 170

TTC AAG GTC TAGGGCAGTT CTAATTTCAA CAGAGAGCAA GCTCTGCCTA CACACCCACA 591
Phe Lys Val

CCAATTCCCT TATATCATCT CTGCTGTTTT TCCTTGAAAT TATTATGAAG CTCACATGGA  651

CAAGGAAGCA AGTATGAGGA TTCACTCAGG ATATCAGTAT ATTCTGTGGT GGCTGTAACC  711

TAAAGGCTCA GAGAACAAAA ATAAAATGTC ATCAAC                             747
```

(SEQ ID NO: 1).

A predicted amino acid sequence is as follows:

```
Met Leu Pro Met Thr Leu Cys Arg Met Ser Trp Met Leu Leu Ser Cys
 1               5                  10                  15

Leu Met Phe Leu Ser Trp Val Glu Gly Glu Glu Ser Gln Lys Lys Leu
            20                  25                  30

Pro Ser Ser Arg Ile Thr Cys Pro Gln Gly Ser Val Ala Tyr Gly Ser
        35                  40                  45

Tyr Cys Tyr Ser Leu Ile Leu Ile Pro Gln Thr Trp Ser Asn Ala Glu
    50                  55                  60
```

```
Leu Ser Cys Gln Met His Phe Ser Gly His Leu Ala Phe Leu Leu Ser
 65                 70                  75                  80

Thr Gly Glu Ile Thr Phe Val Ser Ser Leu Val Lys Asn Ser Leu Thr
                85                  90                  95

Ala Tyr Gln Tyr Ile Trp Ile Gly Leu His Asp Pro Ser His Gly Thr
            100                 105                 110

Leu Pro Asn Gly Ser Gly Trp Lys Trp Ser Ser Ser Asn Val Leu Thr
        115                 120                 125

Phe Tyr Asn Trp Glu Arg Asn Pro Ser Ile Ala Ala Asp Arg Gly Tyr
    130                 135                 140

Cys Ala Val Leu Ser Gln Lys Ser Gly Phe Gln Lys Trp Arg Asp Phe
145                 150                 155                 160

Asn Cys Glu Asn Glu Leu Pro Tyr Ile Cys Lys Phe Lys Val
                165                 170
```

(SEQ ID NO:2).

These sequences were determined from nucleic acids isolated from hamster, but it is believed that other mammalian species will contain INGAP genes which are quite similar. For example, one would expect homologous genes to contain at least about 70% identity. Closer species would be expected to have at least about 75%, 80%, or even 85% identity. In contrast, other family members of the calcium dependent C-type lectins contain at most 60% identity with INGAP. INGAP genes can be isolated from other mammals by utilizing the nucleotide sequence information provided herein.

It has been found that INGAP and fragments thereof are capable of inducing and stimulating islet cells to grow. Moreover, they are capable of inducing differentiation of pancreatic duct cells into insulin-producing ß-cells. Thus many therapeutic modalities are now possible using INGAP, fragments thereof, and nucleotide sequences encoding INGAP. Therapeutically effective amounts of INGAP are supplied to patient pancreata, to isolated islet cells, and to encapsulated pancreatic islet cells, such as in a polycarbon shell.

Known conditions, which can be treated with INGAP, include diabetes mellitus, both insulin dependent and non-insulin dependent, pancreatic insufficiency, pancreatic failure, etc. Inhibition of INGAP expression can be used to treat nesidioblastosis.

However, no data have been reported on the possible effect of INGAP or INGAP peptide in the nervous system.

It would be highly desirable to be provided with a compound derived from an INGAP peptide for repair and/or regeneration of peripheral nervous system.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a compound derived from an INGAP peptide for repair and/or regeneration of peripheral nervous system.

According to the present invention, it has now been found that a small portion of INGAP is sufficient to confer a biological activity on peripheral nervous system.

A fragment of 15 amino acids of the sequence of SEQ ID NO: 2, from amino acid 104-118 is sufficient to stimulate repair and/or regeneration of peripheral nervous system.

More precisely, such an INGAP peptide has the following amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3).

In accordance with the present invention there is provided a neuritogenic compound, which comprises the amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3), functional derivatives and/or fragments thereof and functional peptidometics thereof.

In accordance with the present invention there is provided use of the neuritogenic compound for enhancing neurite outgrowth (*in vitro* or *in vivo*), and more precisely such as for repair and/or regeneration of peripheral nervous system.

In accordance with the present invention there is also provided a neuritogenic pharmaceutical composition, which comprises a therapeutically effective amount of a compound of the present invention in association with a pharmaceutically acceptable carrier.

In accordance with the present invention there is provided use of the neuritogenic composition for enhancing neurite outgrowth (*in vitro* or *in vivo*), and more precisely such as for repair and/or regeneration of peripheral nervous system.

In accordance with the present invention there is also provided a method for repair and/or regeneration of peripheral nervous system in a patient, which comprises administering to said patient a therapeutically effective amount of a neuritogenic compound or of a neuritogenic composition of the present invention.

The patient may be suffering from a neuropathy, included but not limited to diabetic neuropathy, and/or a peripheral nervous system injury.

For the purpose of the present invention the following terms are defined below.

The expression "functional derivatives and/or fragments thereof and functional peptidometics thereof" is intended to mean any isomer, peptide analogues, peptide derivatives including deletion, substitution, or peptidomimetics which retain the neuroregenerative activity of the INGAP peptide has the following amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3).

Except as otherwise expressly defined herein, the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 1972, **11**:1726-1732).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the effect of the INGAP peptide of the present invention on neurite outgrowth in explanted C57BL/6 dorsal root ganglia (DRG).

Fig. 2 illustrates the effects of the INGAP peptide of the present invention, 17-β-estradiol and FK506 on the long term viability of DRG explant cultures.

Fig. 3 illustrates the dose-dependent stimulation of neurite outgrowth from explanted DRG by INGAP peptide at 4 and 7 days in culture. DRG were dissected from 1-month-old C57/B16 mice and cultured in Matrigel in RPMI 1640 tissue culture media (37°C, 5% CO_2) containing 1% antibiotic in the absence or presence of drug treatments. Magnification was obtained with 4X and 1OX (shown) objectives. Representative samples from three independent experiments in which the group size was n = 4-5 are shown. (A) Overall time course of neurite outgrowth observed at 1, 4, and 7 days. (B) Constitutive neurite

outgrowth from DRG after 7 days. (C-E) INGAP peptide treatment enhances the overall density and length of neurites in a dosedependent manner after 4 days in culture. Significant effects are observed at doses of 500 ng/mL (D) and 1000 ng/mL (E). No effects are observed at a concentration of 100 ng/mL (C). Differences from non-treated controls were considered significant where $P < 0.05$.

Fig. 4 illustrates the effect of differentiating agents on INGAP peptide-stimulated neurite outgrowth. Explanted DRGs were cultured as described in the legend to Fig. 1 and treated with INGAP peptide (1000 ng/mL), NGF (50 ng/mL), anti-NGF antibody, or 17-(β-estradiol (10 nM). (A-C, G) INGAP peptide, NGF or 17-(β-estradiol produces strong neurite outgrowth relative to non-treated cultures ($P < 0.05$). (E) Combination of NGF with INGAP peptide enhances neurite outgrowth in comparison to INGAP peptide treatment alone ($P < 0.05$). (F) Preincubation of DRG cultures with anti-NGF antibody leads to marked reduction of INGAP peptide-stimulated neurite outgrowth ($P < 0.05$). (H) No significant enhancement of the INGAP peptide effect on neurite outgrowth is seen when applied with 17-(β-estradiol ($P < 0.05$). Differences from non-treated controls or INGAP peptide-treated cultures were considered significant where $P < 0.05$.

Fig. 5 illustrates the enhancement of mitochondrial activity by INGAP peptide in explanted DRG. Explanted DRG cultures were created as described in the legend to Fig. 1. After 24 h in culture, no significant effects are obtained with INGAP peptide applied in a concentration range of 100-1000 ng/mL. At 4 days in culture, a significant increase in mitochondrial activity indicated by greater MTT reduction is observed in DRGs treated with INGAP peptide at a dose of 1000 ng/mL. In longer-term cultures (7 days), INGAP peptide applied at 500 and 1000 ng/mL continue a trend toward enhanced cell viability ($P > 0.05$). Data represent the means ± SEM of three independent experiments in which the sample size was n = 4-5. Differences from non-treated controls were considered significant where $P < 0.05$.

Fig. 6 illustrates the dose-dependent induction of cell proliferation by INGAP peptide. DRG explant cultures were created as described in the legend to Fig. 1. After 4 days in culture, [^{3}H] thymidine incorporation was assessed in DRG treated with INGAP peptide (100-1000 ng/mL) or high-dose serum (20%). High-dose INGAP peptide (1000 ng/mL) or serum produce strong increases (approximately 4.5- and 5-fold, respectively) in cell proliferation as indicated by

increased [^{3}H] thymidine incorporation. 500 ng/mL INGAP peptide treatment produces less cell proliferation after 4 days (3-fold), while low-dose INGAP peptide (100 ng/mL) has no significant effects relative to non-treated cultures. Data represent means ± SEM. Differences were considered significant where $P < 0.05$.

DETAILED DESCRIPTION OF THE INVENTION

Islet-neogenesis-associated protein, INGAP, is a 175 amino acid pancreatic acinar protein that stimulates pancreatic duct cell proliferation *in vitro* and islet neogenesis *in vivo*. To date, the mitogenic activity of INGAP has been identified only in nonneural tissues. The present invention provides evidence that a pentadecapeptide of INGAP (INGAP peptide) acts as a mitogen in the peripheral nervous system (PNS), and that it enhances neurite outgrowth from DRGs *in vitro* in a time- and dose-dependent manner. The neuritogenic action of INGAP peptide correlates with an increase in [^{3}H] thymidine incorporation ($P < 0.0001$) and mitochondrial activity ($P < 0.001$). Results from these studies suggest that INGAP peptide promotes Schwann cell proliferation in the DRG which releases trophic factors that promote neurite outgrowth.

A 15-amino-acid sequence (amino acids 104-118) contained within the native protein was identified as the biologically active core of INGAP. *In vitro*, the 175-amino-acid protein with molecular mass of approximately of 20 kDa is capable of initiating pancreatic duct cell proliferation, a process that is essential for the neogenesis of islets from precursor cells located in the ductal epithelium. Studies with streptozotocin-induced diabetic hamsters and C57BL/6J mice showed that INGAP treatment is able to ameliorate hyperglycemia in a dose-dependent manner or reverse it completely.

This 15-amino-acid region (INGAP peptide) has been manufactured by solid-phase peptide synthesis and shown to be a potent inducer of islet-cell neogenesis from cells associated with the ductal epithelium, leading to new islet formation in the normal, adult mouse, and hamster.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Using male C57BL/6 mice, dorsal route ganglia (DRG) explant cultures were established to examine the effect of the INGAP peptide of the present invention on neurite outgrowth (Fig. 1). DRG (L3, L4) were dissected from male C57BL/6 mice, and DRG explants were cultured in 14 uL of Matrigel in 2 ml of RPMI 1640 media + 1% antibiotic.

Our studies in this *in vitro* model of the peripheral nervous system suggest that INGAP peptide is effective, in a dose-dependent fashion, as a neurite-promoting agent. This effect is comparable to that observed with a more conventional neurotrophic agent, such as nerve growth factor (NGF).

EXAMPLE 2

Using DRG explant cultures, we examined the effects of the INGAP peptide of the present invention, as well as other agents, on DRG cell viability using the MTT assay (Fig. 2). DRG (L3, L4) were dissected from Balb/C mice, and DRG explants were cultured in 14 uL of Matrigel in 2 ml of RPMI 1640 media + 1% antibiotic. After 48 hours, INGAP, β-estradiol, and/or FK506 were added to the explant cultures as indicated. MTT assay was performed 7 days after the addition of drugs. Data were analyzed using one-way ANOVA with post-hoc Tuckey" test. Differences were consider significant where $p<0.05$. (n=3-4 per treatment group).

The data suggest that INGAP has a potent effect on cell viability. However, the first bar of Fig. 2 represents the viability of cells under normal conditions of culture (14μl of Matrigel in 2 ml RPMI 1640 + 1% Penicillin/streptomycin). A tremendous increase in MTT reduction is seen with the addition of INGAP (doses tested were between 100 ng/mL to 1,500 ng/mL). Maximal effect was observed at 500 ng/mL and did not significantly change with 1000 0r 1,500 ng/mL (the second bar in Fig. 2 refers to 500 ng/mL of INGAP in the medium) to represent a mitogenic effect on Schwann cells. This effect would lead as well to the release of known neurotrophins, including IGF-1, IGF-2, NT-3, PDGF-B, and NGF, that could be directly responsible for inducing the neurite outgrowth demonstrated in Example 1.

This is novel information pointing to the possibility that INGAP treatment could be useful in diabetes not only because of being neogenetic but also neurotrophic.

EXAMPLE 3

MATERIALS AND METHODS

All animal work was performed according to CCAC (Canadian Council on Animal Care) guidelines, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Matrigel extracellular matrix was obtained from Becton-Dickinson U.S.A. Cell culture media (RPMI 1640 without phenol-red) was from Gibco-BRL (Life Technologies, Grand Island, NY). Tissue culture plates were from Falcon (Becton-Dickinson, Franklin Lakes, NJ). 17-R-Estradiol and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). NGF was obtained from Cedar Lane Laboratories (Hornsby, Ontario, Canada), and INGAP synthetic pentadecapeptide was obtained from the Sheldon protein synthesis facility at McGill University (Montreal, Quebec, Canada).

DRG (L3, L4, L5) were dissected from wild-type C57/B16 mice <, and DRG explants were cultured in 7 pL of Matrigel at 37°C (5% CO_2) for 24 h in phenol-red free RPMI 1640 media-free supplemented with 1% penicillin/streptomycin. Neurite outgrowth was followed for 7 days and was visualized with an OLYMPUS™ BX51 system microscope equipped with a Dage-MTI CCD300-RC camera coupled to a Powermate™ 8100 Pentium™ III computer (NEC Corp.) with 128 MB RAM. Bright-field microscope images were stored in uncompressed tagged image file format (TIFF) at a resolution of 600 d.p.i. and analyzed using MCID-M5+ 5.1 image analysis software (Imaging Research Inc.) and SYSTAT 9 statistical software (SPSS Inc.).

Drug treatments

In the neurite outgrowth assays, INGAP (1001000 ng/mL), 17-(β-estradiol (10 nM) or NGF (50 ng/mL) and anti-NGF (1 pg/pL that completely blocks neurite outgrowth of 50 ng/mL of NGF) were added to the serum-free culture media for different times (1-7 days).

For cell viability assays, MTT was dissolved in phosphate buffered saline (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl) and applied to

DRG explant cultures at a concentration of 1.2 mM for 2 h at (37°C, 5% CO_2). Media were then aspirated, and each DRG explant was incubated in 200 pL of DMSO for 1 h. 100 pL of DMSO from each explant was transferred to a 96-well plate and absorbance at 595 nm was read on a Bio-Rad Benchmark plate reader.

For [3H]thymidine incorporation assays, serum-starved DRG explants cultured in Matrigel were exposed to Ingap (100-1000 ng/mL) for 48 h. 1 p Ci/ml of [3H]thymidine was added to each DRG and kept for an additional 48 h, after which time the DRG were washed four times with ice-cold trichloroacetic acid (10%). Ganglia were lysed on ice in 100 pL of tissue solubilizer (NCS-II, Amersham). Lysates were transferred to individual 4 mL scintillation counting vials, and 4 mL of liquid scintillation counting cocktail (Fischer SX25-5 ScintiSafe Plus 50%) was added to each lysate. Counting was performed on a Wallac 1410 liquid scintillation counter.

In all experiments, statistical significance was determined using one-way ANOVA and post-hoc Dunnett's test. The experimental group size was n = 4-5, and differences were considered significant where $P < 0.05$.

RESULTS

Neurite outgrowth

Neurite outgrowth from explanted DRG cultures was assessed at 1, 4, and 7 days in culture (Fig. 3). After 24 h, minor outgrowth was observed, with no significant differences detected between INGAP-treated DRGs and non-treated ones, regardless of the drug concentration ($P > 0.05$, Fig. 3A). After 4 days in culture, significant enhancement of the overall length and density of neurite outgrowth was observed with INGAP treatment at concentrations of 500 ng/mL ($P < 0.001$, Fig. 3D) and 1000 ng/mL ($P < 0.0001$, Fig. 3E) compared to outgrowth from non-treated DRG (Fig. 3B). The lowest dose of INGAP tested, 100 ng/mL, did not have a significant effect on neurite outgrowth ($P > 0.05$, Fig. 3C) at any time point examined. Following 7 days in culture, only INGAP applied at the concentration of 1000 ng/mL continued to significantly enhance neurite outgrowth compared to outgrowth from non-treated DRG ($P < 0.001$, Fig. 3A).

The optimal dose of INGAP (1000 ng/mL) (Fig. 4B) stimulates neurite outgrowth to an extent similar to that of NGF (50 ng/mL) (Fig. 4C, $P > 0.0001$) or 17-β-estradiol (10 nM) (Fig. 4G, $P < 0.05$) at both 4 days (Fig. 4I)

and 7 days in culture relative to non-treated controls (Fig. 4A). Enhanced effects on neurite outgrowth are observed when INGAP is applied in combination with NGF (Fig. 4E, $P < 0.05$). In contrast, 17-(β-estradiol in combination with INGAP produces neurite outgrowth that is not significantly different from that observed with either drug alone (Fig. 4H, $P < 0.05$). A strong reduction in INGAP-stimulated neurite outgrowth was observed in cultures pre-incubated with anti-NGF antibody (Fig. 4F, $P < 0.05$). Figure 4I illustrates the differential effects of the two differentiating agents on neurite outgrowth when applied alone or in combination with INGAP.

MTT assay. After 24 h in culture, the doses of INGAP tested (100, 500, 1000 ng/mL) had no significant effect on metabolically active mitochondria in explanted DRG in comparison to non-treated controls ($P > 0.05$) (Fig. 5, Day 1). Significant differences in mitochondria activity of living cells were seen at four days in culture where INGAP at a concentration of 1000 ng/mL led to significantly larger MTT reduction (Fig. 5, Day 4, $P < 0.05$). In longer-term cultures (7 days), INGAP at concentrations of both 500 and 1000 ng/mL showed a similar trend. (Fig. 5, Day 7, $P > 0.05$).

[^{3}H] Thymidine incorporation

After 4 days in culture, INGAP or high-dose serum (20%) produced highly significant increases in [^{3}H] thymidine incorporation relative to non-treated controls (Fig. 6). Serum treatment induces the greatest cellular proliferation, a 4.5-fold increase relative to non-treated controls ($P < 0.0001$). INGAP exerts a dose-dependent effect on cell proliferation, with no significant effect observed at 100 ng/mL ($P > 0.05$). A 3-fold increase in [^{3}H] Thymidine incorporation at 500 ng/mL ($P < 0.05$) and nearly 4-fold increase at 1000 ng/mL ($P < 0.0001$) of INGAP peptide were obtained.

DISCUSSION

Results from these studies provide the first evidence that the INGAP peptide exerts a neurite-promoting effect in the peripheral nervous system. Surprisingly, and in accordance with the present invention, the applicants have found that INGAP peptide at an optimal concentration of 1000 ng/mL stimulates neurite outgrowth from the DRG explants, to an extent similar to that of the prototypical growth factor, nerve growth factor (NGF) or of phonemic steroids such as 17-(β-estradiol).

The results of the present invention demonstrate that INGAP peptide is a strong enhancer of neurite outgrowth from explanted DRG, and the DRG explant model serves well as a model of nerve axotomy. It is possible that INGAP acts directly on neurons to stimulate the nerve regeneration process, and results of MTT assays indicate that INGAP peptide treatment produces an increase in mitochondrial activity in explanted mouse DRG after 4 days in culture. It is possible that INGAP peptide also exerts an effect on the ganglionic Schwann cells. Indeed, results from thymidine incorporation assays suggest that INGAP peptide treatment leads to an increase in the number of Schwann cells in the sensory ganglia.

Among the candidate molecules that could promote neurite outgrowth under employed conditions for explanted DRGs treated with INGAP peptide, are NGF, IGFs and LIF. Indeed, our experiments utilizing anti-NGF show that at least in part, NGF is involved in promoting neurite outgrowth in the INGAP peptide-treated DRG.

In summary, we provide the first evidence that INGAP peptide exerts an effect in the peripheral nervous system, in particular, on the sensory ganglia. We conclude that INGAP peptide enhances neurite outgrowth from explanted DRG cultures by inducing the proliferation of Schwann cells, which may then produce the growth factors required to support nerve regeneration. These findings are of particular relevance to the peripheral neuropathy associated with almost half of the cases of diabetes, in that the INGAP may represent a potential therapeutic that not only restores normoglycemia via islet neogenesis but also ameliorates one of the most common and devastating complications of the disease.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention, following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A neuritogenic compound which comprises the amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3), functional derivatives and/or fragments thereof and functional peptidometics thereof.

2. Use of the compound of claim 1 for enhancing neurite outgrowth.

3. The use of claim 2, wherein said enhancing of neurite outgrowth is for repair and/or regeneration of peripheral nervous system in a patient

4. A neuritogenic pharmaceutical composition, which comprises a therapeutically effective amount of a compound claim 1 in association with a pharmaceutically acceptable carrier.

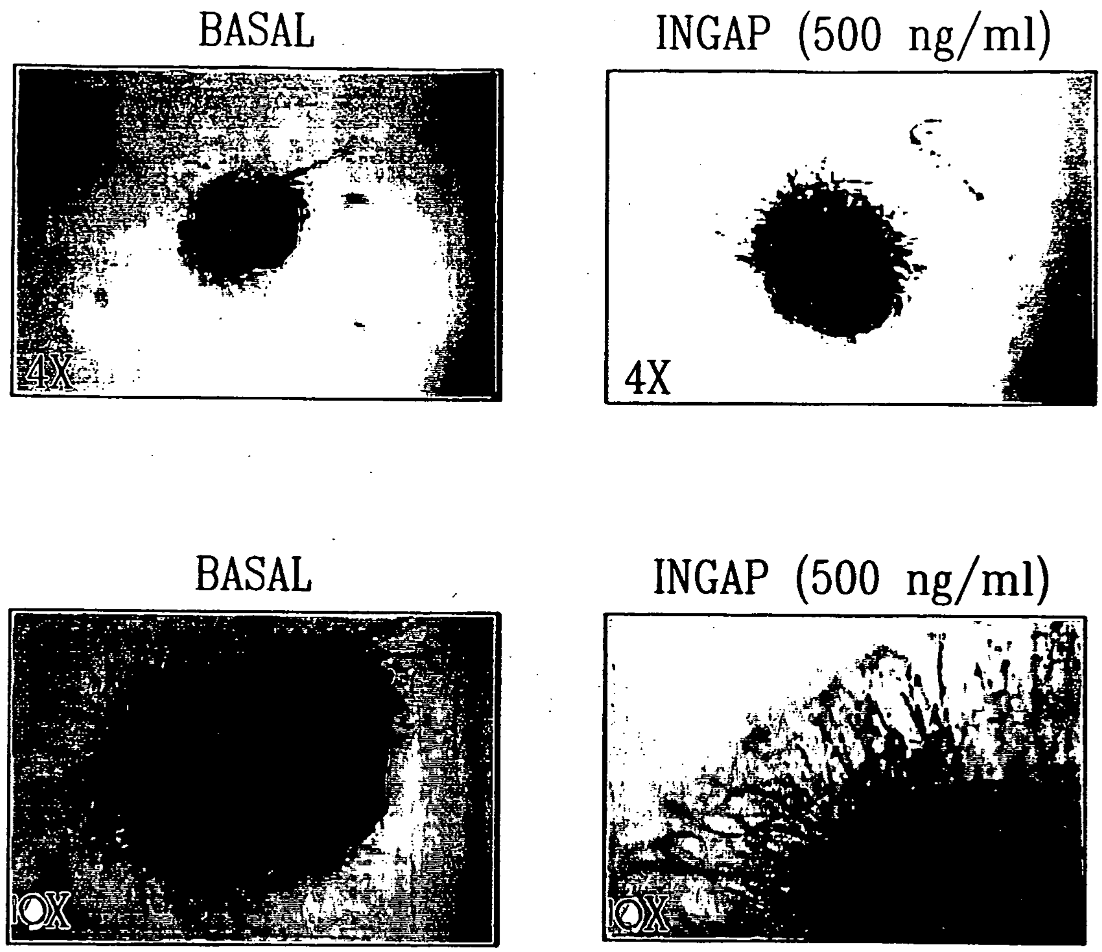
5. Use of the composition of claim 4 for enhancing neurite outgrowth.

6. The use of claim 5, wherein said enhancing of neurite outgrowth is for repair and/or regeneration of peripheral nervous system in a patient

7. A method for repair and/or regeneration of peripheral nervous system in a patient, which comprises administering to said patient a therapeutically effective amount of a compound claim 1 or of a composition of claim 4.

8. The method of claim 7, wherein said patient is suffering from a neuropathy and/or a peripheral nervous system injury.

9. The method of claim 8, wherein said neuropathy is diabetic neuropathy.



DAY	BASAL	100 ng/ml	500 ng/ml	1000 ng/ml	1500 ng/ml
1	–	+	+	+	++
2	–	++	++++	++++	+++++

Fig-1

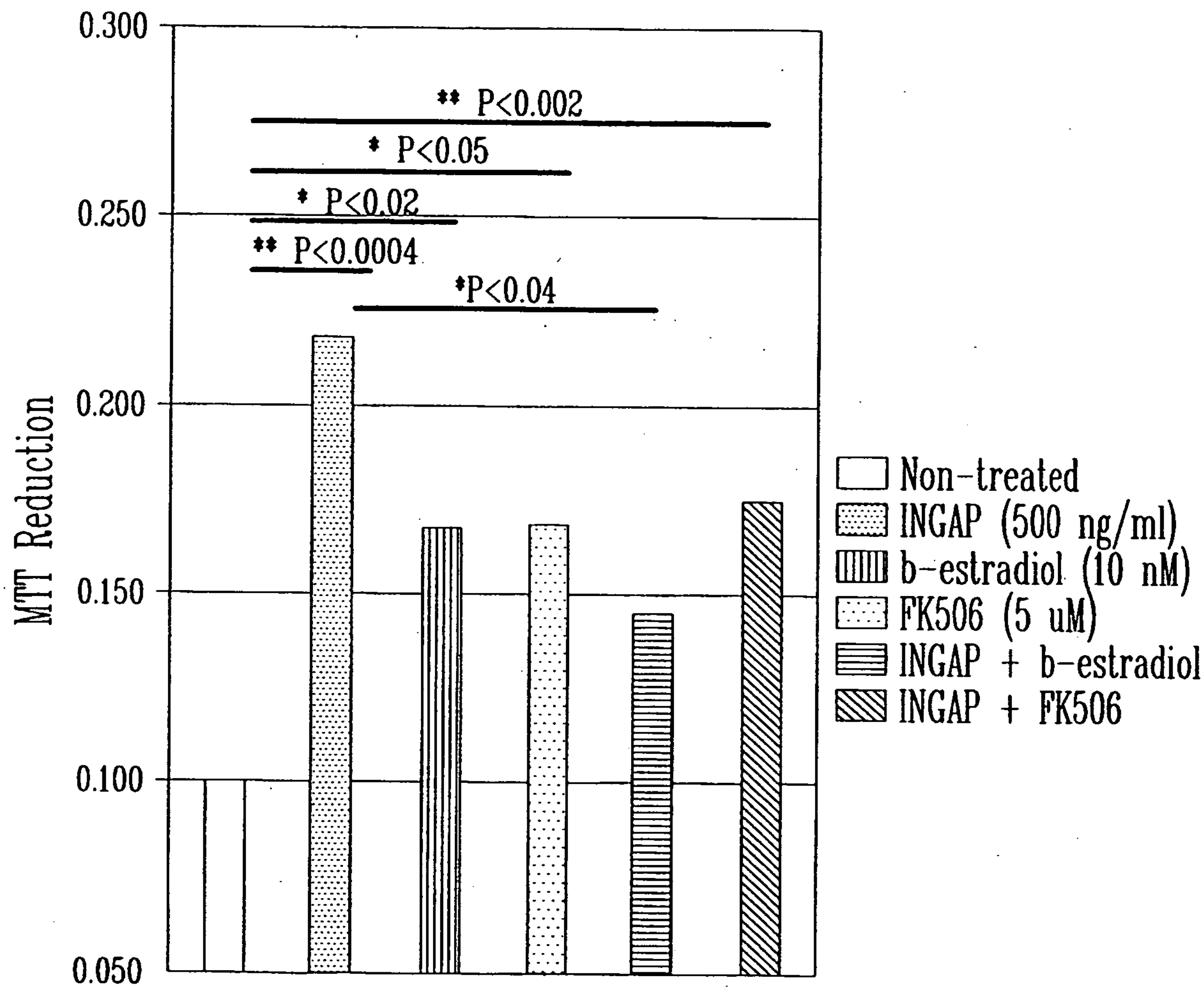

0.300
0.250
0.200
0.150
0.100
0.050
MTT Reduction
** P<0.002
* P<0.05
* P<0.02
** P<0.0004
*P<0.04
Non-treated
INGAP (500 ng/ml)
b-estradiol (10 nM)
FK506 (5 uM)
INGAP + b-estradiol
INGAP + FK506

Fig- 2

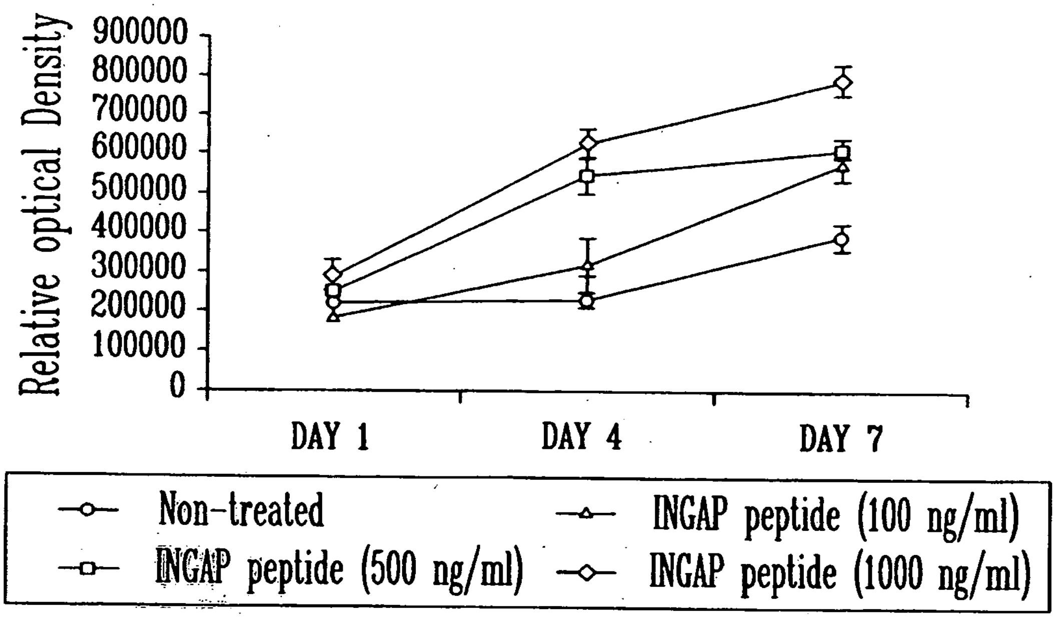


Fig- 3A

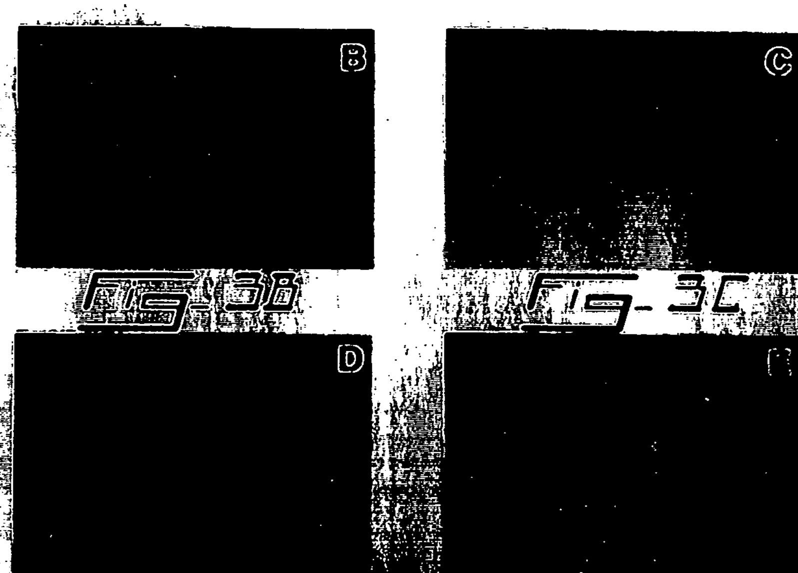


Fig- 3B

Fig- 3C

Fig- 3D

Fig- 3E

Non-treated

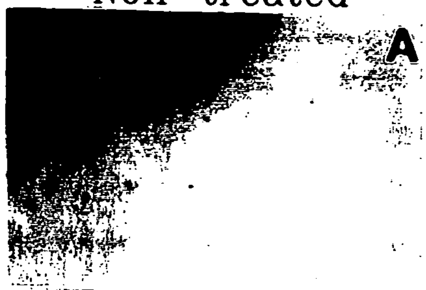


Fig-4A

INGAP peptide

Fig-4B

NGF

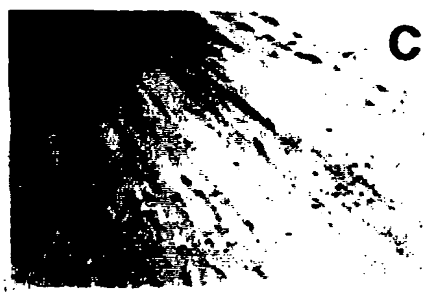


Fig-4C

Anti-NGF

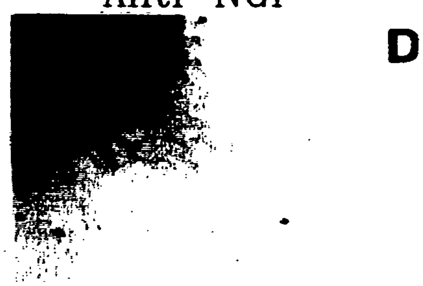


Fig-4D

INGAP peptide + NGF

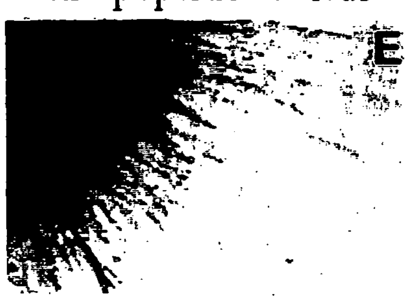


Fig-4E

INGAP peptide + anti-NGF

Fig-4F

ED

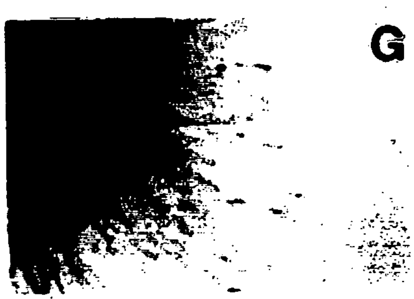


Fig-4G

INGAP peptide + ED

Fig-4H

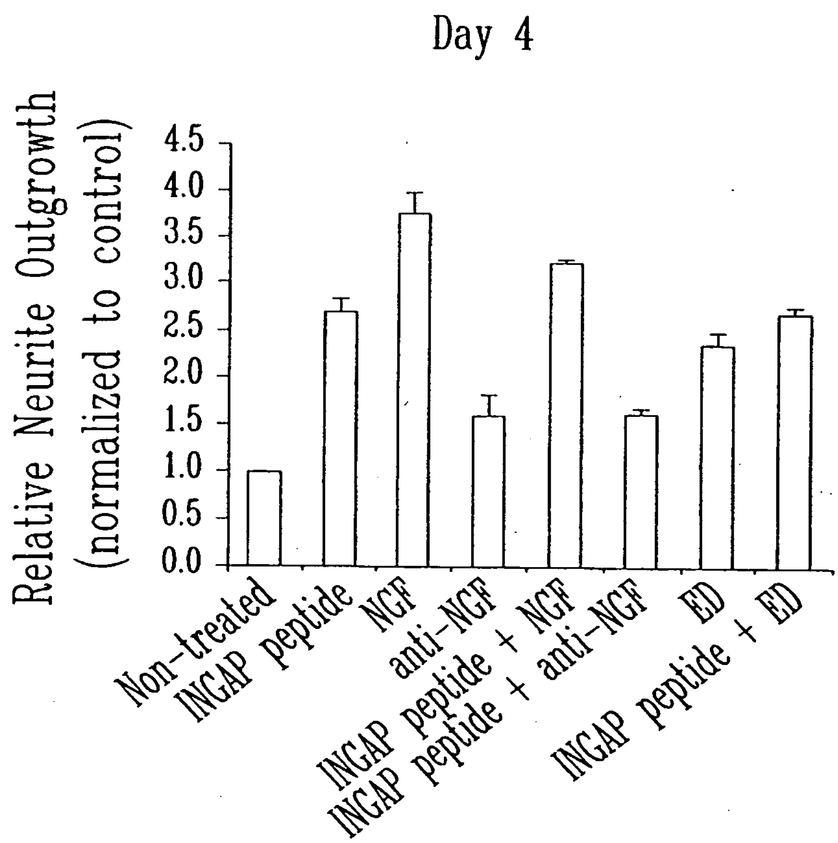

Day 4
Relative Neurite Outgrowth (normalized to control)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Non-treated
INGAP peptide
NGF
anti-NGF
INGAP peptide + NGF
INGAP peptide + anti-NGF
ED
INGAP peptide + ED

Fig-4I

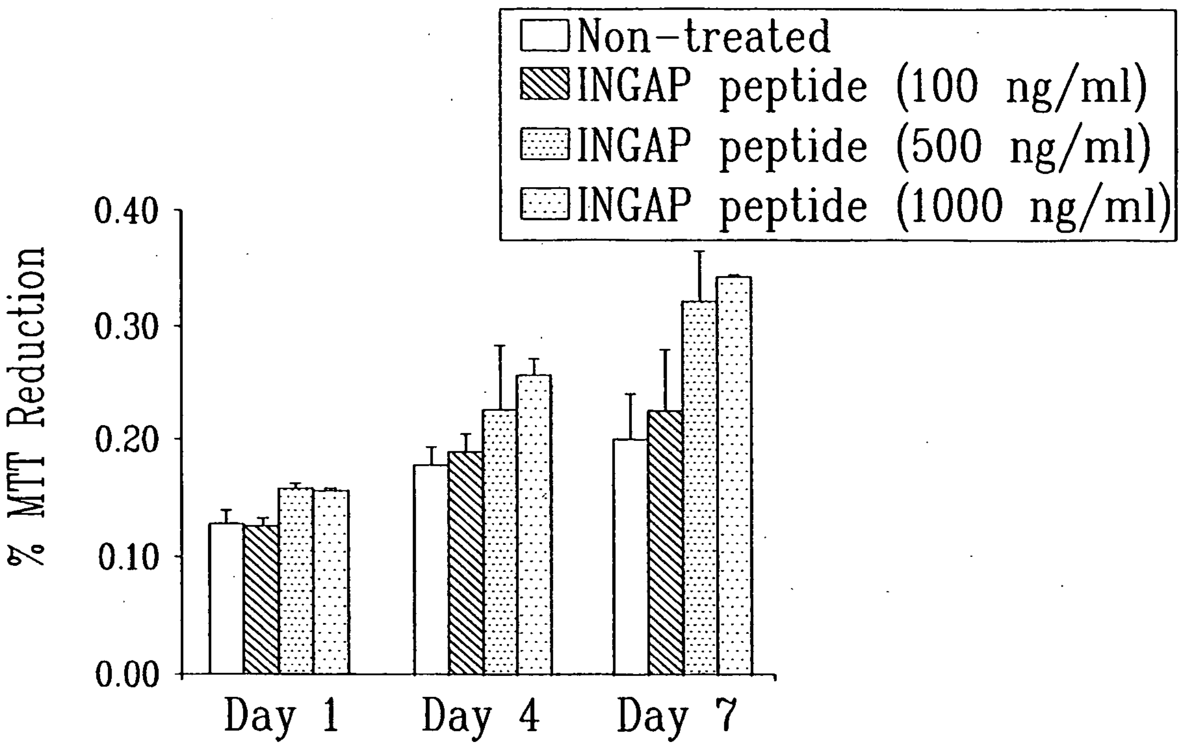

Non-treated
INGAP peptide (100 ng/ml)
INGAP peptide (500 ng/ml)
INGAP peptide (1000 ng/ml)
% MTT Reduction
0.40
0.30
0.20
0.10
0.00
Day 1
Day 4
Day 7

Fig- 5

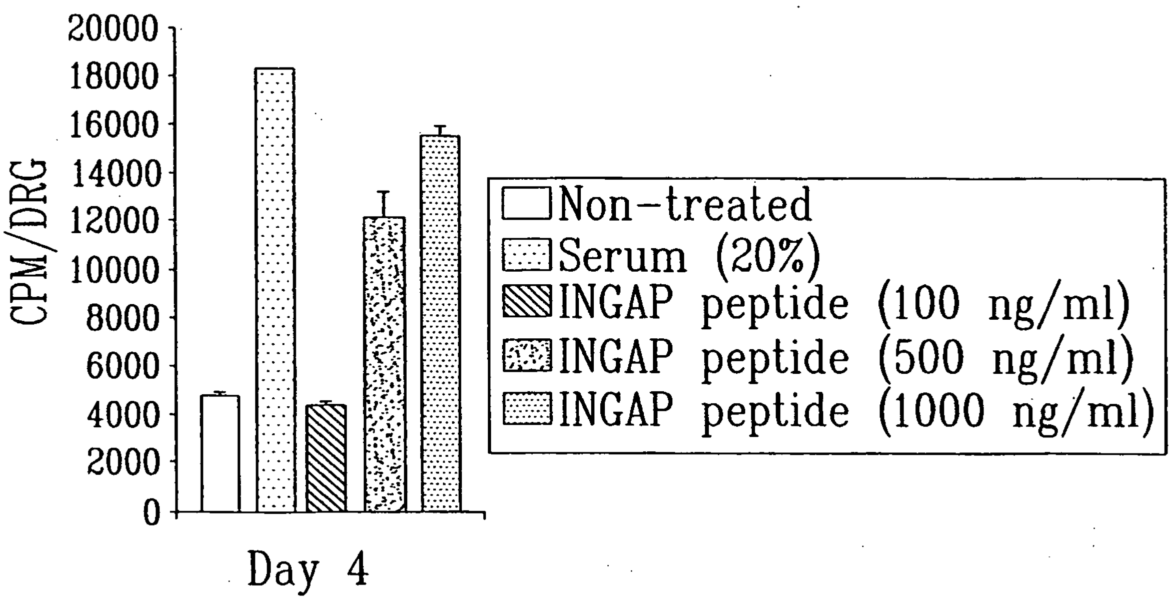

Non-treated
Serum (20%)
INGAP peptide (100 ng/ml)
INGAP peptide (500 ng/ml)
INGAP peptide (1000 ng/ml)
CPM/DRG
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Day 4

Fig- 6

SEQUENCE LISTING

```
<110> McGill University
      Rosenberg, Lawrence
      Maysinger, Dusica

<120> NEURITOGENIC COMPOUND AND USES THEREOF

<130> 1770-286PCT FC

<150> US 60/272,063
<151> 2001-01-01

<160> 3

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 747
<212> DNA
<213> Islet Neogenesis Associated protein (INGAP)

<220>
<221> CDS
<222> (20)...(541)

<400> 1
ctgcaagaca ggtaccatg atg ctt ccc atg acc ctc tgt agg atg tct tgg      52
                     Met Leu Pro Met Thr Leu Cys Arg Met Ser Trp
                      1               5                  10

atg ctg ctt tcc tgc ctg atg ttc ctt tct tgg gtg gaa ggt gaa gaa      100
Met Leu Leu Ser Cys Leu Met Phe Leu Ser Trp Val Glu Gly Glu Glu
            15                  20                  25

tct caa aag aaa ctg cct tct tca cgt ata acc tgt cct caa ggc tct      148
Ser Gln Lys Lys Leu Pro Ser Ser Arg Ile Thr Cys Pro Gln Gly Ser
        30                  35                  40

gta gcc tat ggg tcc tat tgc tat tca ctg att ttg ata cca cag acc      196
Val Ala Tyr Gly Ser Tyr Cys Tyr Ser Leu Ile Leu Ile Pro Gln Thr
    45                  50                  55

tgg tct aat gca gaa cta tcc tgc cag atg cat ttc tca gga cac ctg      244
Trp Ser Asn Ala Glu Leu Ser Cys Gln Met His Phe Ser Gly His Leu
 60                  65                  70                  75

gca ttt ctt ctc agt act ggt gaa att acc ttc gtg tcc tcc ctt gtg      292
Ala Phe Leu Leu Ser Thr Gly Glu Ile Thr Phe Val Ser Ser Leu Val
                80                  85                  90

aag aac agt ttg acg gcc tac cag tac atc tgg att gga ctc cat gat      340
Lys Asn Ser Leu Thr Ala Tyr Gln Tyr Ile Trp Ile Gly Leu His Asp
            95                 100                 105
```

ccc tca cat ggt aca cta ccc aac gga agt gga tgg aag tgg agc agt 388
Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly Trp Lys Trp Ser Ser
110 115 120

tcc aat gtg ctg acc ttc tat aac tgg gag agg aac ccc tct att gct 436
Ser Asn Val Leu Thr Phe Tyr Asn Trp Glu Arg Asn Pro Ser Ile Ala
125 130 135

gct gac cgt ggt tat tgt gca gtt ttg tct cag aaa tca ggt ttt cag 484
Ala Asp Arg Gly Tyr Cys Ala Val Leu Ser Gln Lys Ser Gly Phe Gln
140 145 150 155

aag tgg aga gat ttt aat tgt gaa aat gag ctt ccc tat atc tgc aaa 532
Lys Trp Arg Asp Phe Asn Cys Glu Asn Glu Leu Pro Tyr Ile Cys Lys
160 165 170

ttc aag gtc tagggcagtt ctaatttcaa cagagagcaa gctctgccta 581
Phe Lys Val

cacacccaca ccaattccct tatatcatct ctgctgtttt tccttgaaat tattatgaag 641
ctcacatgga caaggaagca agtatgagga ttcactcagg atatcagtat attctgtggt 701
ggctgtaacc taaaggctca gagaacaaaa ataaaatgtc atcaac 747

<210> 2
<211> 174
<212> PRT
<213> Islet Neogenesis Associated protein (INGAP)

<400> 2
Met Leu Pro Met Thr Leu Cys Arg Met Ser Trp Met Leu Leu Ser Cys
1 5 10 15
Leu Met Phe Leu Ser Trp Val Glu Gly Glu Glu Ser Gln Lys Lys Leu
20 25 30
Pro Ser Ser Arg Ile Thr Cys Pro Gln Gly Ser Val Ala Tyr Gly Ser
35 40 45
Tyr Cys Tyr Ser Leu Ile Leu Ile Pro Gln Thr Trp Ser Asn Ala Glu
50 55 60
Leu Ser Cys Gln Met His Phe Ser Gly His Leu Ala Phe Leu Leu Ser
65 70 75 80
Thr Gly Glu Ile Thr Phe Val Ser Ser Leu Val Lys Asn Ser Leu Thr
85 90 95
Ala Tyr Gln Tyr Ile Trp Ile Gly Leu His Asp Pro Ser His Gly Thr
100 105 110
Leu Pro Asn Gly Ser Gly Trp Lys Trp Ser Ser Ser Asn Val Leu Thr
115 120 125
Phe Tyr Asn Trp Glu Arg Asn Pro Ser Ile Ala Ala Asp Arg Gly Tyr
130 135 140
Cys Ala Val Leu Ser Gln Lys Ser Gly Phe Gln Lys Trp Arg Asp Phe
145 150 155 160
Asn Cys Glu Asn Glu Leu Pro Tyr Ile Cys Lys Phe Lys Val
165 170

<210> 3
<211> 15
<212> PRT

<213> INGAP peptide

<400> 3
Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly
1 5 10 15

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2002 (12.09.2002)

PCT

(10) International Publication Number
WO 02/070551 A3

(51) International Patent Classification⁷: C07K 14/47, A61K 38/17, A61P 25/02

(21) International Application Number: PCT/CA02/00257

(22) International Filing Date: 28 February 2002 (28.02.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/272,063 1 March 2001 (01.03.2001) US

(71) Applicant (*for all designated States except US*): **MCGILL UNIVERSITY** [CA/CA]; 845 Sherbrooke Street West, Montréal, Québec H3A 2T5 (CA).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **ROSENBERG, Lawrence** [CA/CA]; 6507, Fern Road, Côte St-Luc, Québec H4V 1E4 (CA). **MAYSINGER, Dusica** [CA/CA]; 406, Pine Avenue, Montréal, Québec H2W 1S2 (CA).

(74) Agents: **OGILVY RENAULT** et al.; Suite 1600, 1981 McGill College Avenue, Montreal, Québec H3A 2Y3 (CA).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 10 April 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/070551 A3

(54) **Title:** NEURITOGENIC COMPOUND AND USES THEREOF

(57) **Abstract:** The present invention relates to a neuritogenic compound for neurite outgrowth, which comprises the amino acid sequence: Gly Leu His Asp Pro Ser His Gly Thr Leu Pro Asn Gly Ser Gly (SEQ ID NO:3), functional derivatives and/or fragments thereof and functional peptidometics thereof. There is also provided a method for repair and/or regeneration of peripheral nervous system in a patient.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 02/00257

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 A61K38/17 A61P25/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAFAELOFF R ET AL.: "Cloning and Sequencing of the Pancreatic Islet Neogenesis Associated Protein (INGAP) Gene and Its Expression in Islet Neogenesis in Hamsters" JOURNAL OF CLINICAL INVESTIGATION, vol. 99, no. 9, May 1997 (1997-05), pages 2100-2109, XP002173530 page 2105 -page 2108; figures 3A,4	1,4
X	US 5 840 531 A (VINIK ET AL.) 24 November 1998 (1998-11-24) column 1, line 25 -column 5, line 17; figure 2	1,4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 January 2003	24/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schmidt, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	TAM J ET AL.: "Islet-Neogenesis-Associated Protein Enhances Neurite Outgrowth from DRG Neurons" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 291, 1 March 2002 (2002-03-01), pages 649-654, XP002226504 the whole document -----	1-7

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-9 (all partially)

The scope of claims 1-9, in as far as the expression "functional derivatives and/or fragments thereof and functional peptidometics thereof" is concerned, is so unclear (Article 6 PCT) that a meaningful International Search is impossible with regard to this expression.

Moreover, such an expression may comprise a wide range of compounds and is therefore speculative, embracing a great variety of possibilities not yet explored by the Applicant, the effect of which cannot be expected by the skilled person using the teaching disclosed in the current application and his technical knowledge to reproduce without undue burden all the possibilities which are actually claimed. Therefore, subject-matter of claims 1-9 also lacks disclosure (Article 5 PCT).

Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds comprising the amino acid sequence of SEQ ID NO 3.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 02/00257

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 2,3 and 5-9 encompass a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-9 (all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 02/00257

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5840531 A	24-11-1998	US 5834590 A	10-11-1998
		AU 708499 B2	05-08-1999
		AU 4914996 A	11-09-1996
		CA 2213610 A1	29-08-1996
		EP 0815129 A1	07-01-1998
		JP 11500907 T	26-01-1999
		WO 9626215 A1	29-08-1996

Form PCT/ISA/210 (patent family annex) (July 1992)